IMMUNOHISTOCHEMICAL EXPRESSION OF p63 AND β-CATENIN IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA

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BRANCH – VI

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ADHIPARASAKTHI DENTAL COLLEGE & HOSPITAL
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ORAL PATHOLOGY AND MICROBIOLOGY

CERTIFICATE

This is to certify that **Dr. S.PRADEEPSANKAR,** Post Graduate student (2016-2019) in the Department of Oral Pathology and Microbiology, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319, has done this dissertation titled **"IMMUNOHISTOCHEMICAL EXPRESSION OF p63 AND β-CATENIN IN DIFFERENT GRADES OF ORAL SQUAMOUS CELL CARCINOMA"** under our direct guidance and supervision in partial fulfilment of the regulations laid down by **THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY**, Chennai – 600032 for **MASTER OF DENTAL SURGERY - (BRANCH-VI) ORAL PATHOLOGY AND MICROBIOLOGY** degree examination.

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DECLARATION

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I hereby declare that no part of the dissertation will be utilized for gaining financial assistance or any promotion without obtaining prior permission of the Principal, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319. In addition, I declare that no part of this work will be published either in print or in electronic media without the guides who has been actively involved in dissertation. The author has the right to reserve for publishing the work solely with the permission of the Principal, Adhiparasakthi Dental College and Hospital, Melmaruvathur – 603319.

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ABSTRACT

BACKGROUND: Oral squamous cell carcinoma (OSCC) results from the multistep accumulation of heterogenous genetic changes. The poor prognosis of oral cancer is due to several reasons such as late stage diagnosis, low response to current therapeutic strategies, primary site recurrence, high rate of metastasis to regional lymph nodes etc. which has strongly recommended the need to improve the diagnostic capabilities. Assessing the tumor behaviour at the molecular level will help in initiating appropriate management there by reducing the morbidity associated with malignancy. Two important molecular factors that is known to be associated with cell proliferation and maintaining cell junction are p63 and β-Catenin respectively.

AIM: To evaluate the expression of p63 and β-catenin in different grades of oral squamous cell carcinoma using immunohistochemistry.

MATERIALS AND METHODS: A total of 36 samples were examined for the immunohistochemical expression of p63 and β-catenin. The control group includes 15 formalin fixed paraffin embedded tissue blocks of normal buccal mucosa. The study group includes 21 cases of formalin fixed paraffin embedded tissue blocks of oral squamous cell carcinoma (10 cases of well differentiated and 11 case of moderately differentiated squamous cell carcinoma). 3 micron thickness sections were made from each sample and stained with p63 antibody and βcatenin antibody. The intensity and area of staining was assessed and scored. The data obtained statistically analysed.

RESULTS: Comparison of staining intensity showed a significant difference between normal and well differentiated carcinoma and also between normal and moderately differentiated carcinoma. However, no significant difference in staining intensity was found between well differentiated and moderately differentiated carcinoma. Similarly comparison of area of staining showed a significant difference between normal and well differentiated carcinoma and also between normal and moderately differentiated carcinoma. However, no significant difference in area of staining was found between well differentiated and moderately differentiated carcinoma. Comparison of staining intensity did not show any significant difference between normal and well differentiated carcinoma, between normal and moderately differentiated carcinoma and also between well differentiated and moderately differentiated carcinoma. Similarly comparison of area of staining showed no significant difference between normal and well differentiated carcinoma, between normal and moderately differentiated carcinoma and also between well differentiated and moderately differentiated carcinoma

CONCLUSION: We conclude that the expression of both β - catenin and p63 can be used as a prognostic marker in oral squamous cell carcinoma.

KEY WORDS: Squamous cell carcinoma, Immunohistochemistry, β - catenin, p63.

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LIST OF ABBREVIATIONS

DPX	-	Dibutyl Phthalate Xylene
DAB	-	Diaminobenzidine
HRP	-	Horseradish peroxidase
PDGF	-	Platelet Derived Growth Factor
TGF	-	Transforming Growth Factor
WNT	-	Wingless-type integration site family member
EMT	-	Epithelial Mesenchymal Transition
OSCC	-	Oral Squamous Cell Carcinoma
TNM	-	Tumor Lymphnodes Metastasis
CT	-	Computed tomography
MRI	-	Magnetic Resonance Imaging
Wnt	-	Wingless type integration site family member
IHC	-	Immunohistochemistry
APC	-	Antigen presenting cell
EDTA	-	Ethylene Diamine –Tetra – Acetic Acid
PBS	-	Phosphate buffered solution
TBS	-	Tris Buffered solution
EGFR	-	Epidermal Growth factor receptor
MUC1	-	Mucin cell associated 1
SSCP	-	Single Strand conformation polymorphism
TCF	-	T- Cell Factor
MMD	-	Matrix Metalloproteinase
RT-PCR	-	Reverse Transcriptase Polymerase chain reaction
MAPK	-	Mitogen - Activated Protein Kinase

GSK	-	Glycogen Synthase Kinase
ATP	-	Adenosine Triphosphate
ROS	-	Reactive oxygen species
HNSCC	-	Head and Neck Squamous Cell Carcinoma
PLGA	-	Polymorphous Low Grade Adenocarcinoma
ACC	-	Adenoid Cystic Carcinoma

INTRODUCTION

Squamous cell carcinoma is the most common malignancy of Oral cavity[1]. It is defined as a "Malignant epithelial neoplasm exhibiting squamous differentiation as characterized by the formation of keratin and the presence of intercellular bridges[2]. It is the 8th most common cancer in the world and 3rd most common malignancy in south Asia with epidemiologic variation between different geographic regions[2,]. The age of individuals diagnosed with oral squamous cell carcinoma in developed countries is more than 60years, whereas in developing countries it is between the 4th to 6th decades and shows a tendency towards male predominance[3,4].

Oral squamous cell carcinoma (OSCC) results from the multistep accumulation of heterogenous genetic changes[4,5]. Important risk factors for OSCC include the use of tobacco or betel quid chewing, alcohol consumption, human papilloma virus and poor nutrition[5-8]. Most frequent sites affected by OSCC are buccal mucosa, tongue and palate[8]. The clinical staging is done based on the tumour size, regional lymph node involvement and metastasis (TNM Staging). Computed Tomography (CT) and Magnetic Resonance Imaging (MRI) are used to assess the extent of bone and soft tissue involvement and to ascertain the prognosis[9,10].

Histopathology remains the "Gold Standard" procedure to diagnose OSCC till date and OSCC is graded histopathologically[10]. Based on the proportion of the neoplasm resembling normal squamous epithelium and the amount of keratin production, lesions are classified into three distinct grades as well, moderate and poorly differentiated carcinoma[1]. The poor prognosis of oral cancer is due to several reasons such as late stage of diagnosis, low response to current therapeutic strategies, primary site recurrence, high rate of metastasis to regional lymph nodes etc. This has strongly recommended the need to improve the diagnostic capabilities for early detection and managenent[11,12].

One of the recent advances in the diagnosis of OSCC is Immunohistochemistry (IHC). It aids in histopathological analysis by detecting gene expression at the protein level[13]. Immunohistochemistry is defined as the technique for identifying cellular or tissue constituents (antigens) by means of antigen-antibody interaction. The site of antibody binding is identified either by direct labelling of antibody or by use of secondary labelling method[14]. This is used to visualize both normal and diseased states of tissues, infectious agent and other component that may not be demonstrated by histochemical or special stain[15].

The color of the reaction is determined by the selection of a precipitating chromogen, usually diaminobenzidine (brown) or aminoethylcarbazole (red) with which the enzyme reacts. Use of

positive and negative controls is of paramount importance and should be a routine in immunohistochemistry[15]. Negative controls confirm that the staining being reported is due to the antibody binding and positive controls confirm that the antibody is working and that the suspected target of antibody is the acted target[14,15]. IHC panel using multiple prognostic molecular biomarkers can provide information to identify high risk patients, thus playing an important role in early diagnosis and treatment[15,16].

Cell cycle is a highly regulated process and involves many molecules and interrelated pathways. Mutations of the genes regulating the cell proliferation leads to the development of carcinoma. The grade of differentiation along with the extent of invasion and metastasis largely determines the prognosis of the patient. For metastasis to occur, firstly the cell has to severe its attachment with the adjoining cells. Assessing the tumor behaviour at the molecular level will help in initiating appropriate management there by reducing the morbidity associated with malignancy. Two important molecular factors that is known to be associated with cell proliferation and maintaining cell junction are p63 and β- Catenin respectively[17].

p63 is a member of p53 family which is located on chromosome 3q27-29[16]. It has been found to play a role in the development of epithelial tissue, epithelial stem cell maintenance and differentiation and thus it is vital for cell cycle regulation[18]. Hence mutation of p63

gene will lead to dysregulation of cell proliferation and stratification thus contributing to carcinogenesis. In many different tumour types an overexpression of mutated p63 has been observed[19,20]. However its expression in OSCC has not been fully explored. Hence we decided to select p63 as one of the IHC marker and analyse its expression in different grades of OSCC.

β-Catenin is an important component of cell adhesion complex and helps in maintaining the structural integrity and organisation of stratified squamous epithelium. It also acts as an transcriptional factor in Wnt signalling pathway[21,22]. The free cytoplasmic accumulation of β-Catenin is regulated by APC gene thus inhibiting transcription of factors essential for cell proliferation[22]. Down regulation of β-Catenin at the cell junction leads to detachment of the cells from the adjoining cells and increased transcription induced by β- Catenin favours cell invasion and metastasis. Aberrant expression of β-Catenin have been identified in several human cancers[23, 24]. However the expression of β-Catenin in OSCC have not been fully explored. Hence we decided to perform an IHC study to observe the expression of β- Catenin in different grades of OSCC.

With this background we undertook the current IHC study to analyse the expression of p63 and β-Catenin in different grades of OSCC.

AIM & OBJECTIVES

AIM :

To evaluate the expression of p63 and β-catenin in different grades of oral squamous cell carcinoma using Immunohistochemistry.

OBJECTIVES OF THE STUDY:

1. To analyse the intensity of p63 expression in normal mucosa, well differentiated and moderately differentiated oral squamous cell carcinoma.
2. To analyse the intensity of β-catenin expression in normal mucosa, well differentiated and moderately differentiated oral squamous cell carcinoma.
3. To analyse the area of staining of p63 in normal mucosa, well differentiated and moderately differentiated oral squamous cell carcinoma.
4. To analyse the area of staining of β-catenin expression in normal mucosa, well differentiated and moderately differentiated oral squamous cell carcinoma.
5. To compare the expression of p63 and β-catenin in well differentiated and moderately differentiated oral squamous cell carcinoma.

REVIEW OF LITERATURE

β-CATENIN

The word catenin is derived from latin word "Catena" which means Chain. β- Catenin was first discovered in 1980s as a component of E-Cadherin mediated cell adhesion complex. They link the E-Cadherin to actin cytoskeleton through α-catenin. β-Catenin is also a key regulatory protein in Wnt mediated signal transduction pathways. It is encoded by gene CTNNB located in chromosome 3p22[24].

β-Catenin is normally found along the cell membrane. APC is a tumour suppressor gene which prevents the accumulation of β- Catenin in the cytoplasm by forming macromolecular complex and its degradation by ubiquitin – proteosome pathway[21,22]. Wnt signalling blocks APC and allows translocation of β- Catenin from cytoplasm to nucleus. In the nucleus β- Catenin forms a complex TCF, a transcription factor and upregulates cell proliferation[24].

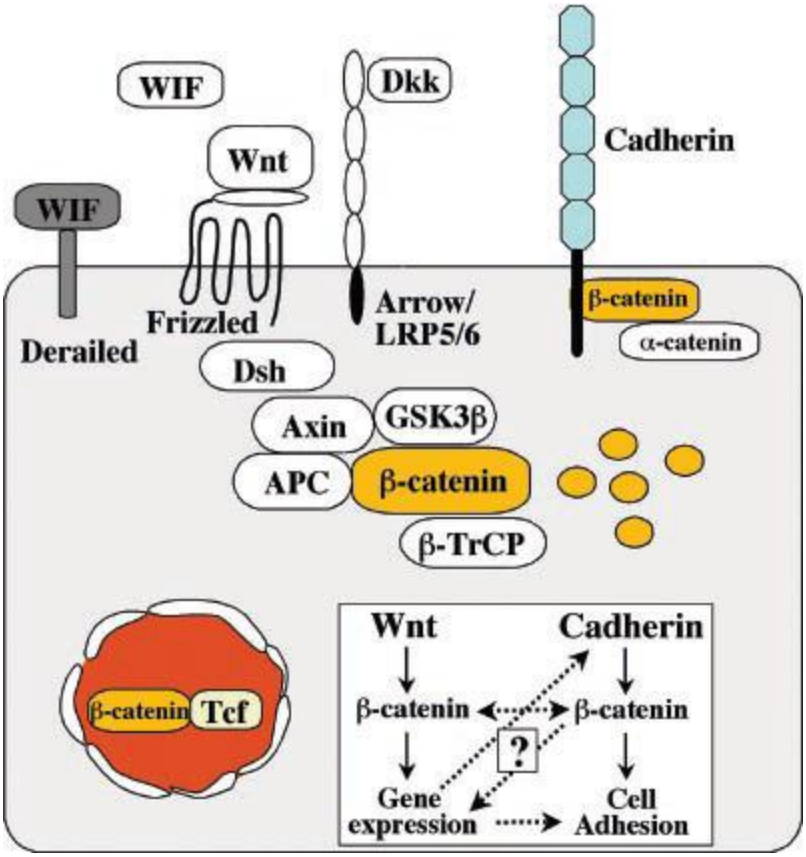
It consists of an amino terminal domain with 130 amino acids, carboxy terminal domain with 100 amino acids and also contains 42 amino acids in 12 imperfect repeats called arm repeats. Each arm repeat form 3 helixes arranged in the form of triangles. The arm repeat (5-9) is the core binding sites of TCF , cadherin and APC and the arm repeat (3-4) serves as the binding site for TCF, APC and Axin. The

first arm repeat and the arm repeat 11 C- Terminal is the region for the transactivation of Wnt target genes[17].

The N and C terminal domains are negatively charged, sensitive to trypsin, structurally flexible and also act as 'intramolecular chaperons' of arm repeat domain. They increase the binding specificity of arm repeats and also prevents its self-aggregation. N terminal domain (NTD) is the binding site of α catenin, GSK3 and CSK-I phosphorylation sites which is recognized by β- transducing containing proteins ubiquitin ligase (β-TrCP)[21]. Helix-C caps the hydrophobic surface of the carboxy terminal end of the arm repeat and recruits transcriptional coactivators and activates Wnt responsive genes. (Figure 1)

β-Catenin is essential for maintaining the structural integrity and organization of stratified squamous epithelium[24]. Stabilization of β-Catenin in the cytoplasm leads to increased nuclear transcriptional activity. This is observed as aberrant expression of β-Catenin in many malignancies.

Figure 1: β- Catenin in Wnt signaling and the cadherin complex



β–CATENIN IN ORAL SQUAMOUS CELL CARCINOMA

Claudia Bagutti *et al* (1998)[25] compared the expression of E- and P-cadherin with five different integrin subunits and with *α, β, and γ* catenin .They stained a panel of oral SCCs with ten different antibodies, using double-label immunofluorescence. They found a reduced expression of cadherins and integrins in poorly differentiated tumours, while reduced catenin expression was seen in all tumours, regardless of differentiation status.

Gasparoni *et al* (2002)[26] analyzed the subcellular localization of β-catenin in cultures of human oral normal and malignant (cell lines SCC15 and SCC25) keratinocytes. Membranous β-catenin localization appeared in normal cells and decreased progressively in SCC15 & SCC25 Cells. It was mostly cytoplasmic and nuclear in SCC 25 cells. In the growth assays, SCC25 cell lines proliferated faster than in normal and SCC15 cells over a period of 6 days. Carcinoma sections showed a combination of membranous and cytoplasmic staining while a few invading epithelial islands of tumors showed nuclear localization of β-catenin.

Agnes Bankfalvi *et al* (2002)[27] studied about the deranged expression of the E-cadherin/ β- catenin complex and the epidermal growth factor receptor in the clinical evolution and progression of oral squamous cell carcinomas using immunohistochemistry. All three

molecules were constitutionally expressed in the basal/parabasal layers of tumour adjacent 'normal' epithelium, in contrast to a significant increase of EGFR and heterogeneous expression of E-cad/β-cat in dysplasia. In OSCCs, overexpression of EGFR correlated significantly with lower tumour grade and poor prognosis, loss of E-cad was a significant marker for shortened survival, reduced β-cat staining was a predictive marker for lymph node metastasis.

N. Tanaka *et al* (2003)[28] investigated the immunohistochemical expression of E-cadherin, α-catenin and β-catenin in oral squamous cell carcinoma and examined the correlation between their expressions and the presence of regional lymph node metastasis. They found a significantly greater reduction in expression levels of E-cadherin, α-catenin and β-catenin in the metastatic group compared to the nonmetastatic group. However, there was no significant correlation between their expressions and the features of the regional metastasis, the number of metastatic lymph nodes or the presence of extracapsular metastasis. They concluded that evaluation of the immuno-histochemical expression of E-cadherin, α-catenin and β-catenin is extremely valuable for the diagnosis of metastatic occurrence.

Stephan *et al* (2004)[29] performed an immuno-histochemical double staining study to determine the expression and sub-cellular distribution of MUC1 and Nuclear β-Catenin in patient with colorectal carcinoma. They found that MUC1 was strongly expressed in tumor

center and at the invasive front. Nuclear accumulation of β-catenin was found at the invasive tumor front. They concluded the nuclear co-expression of MUC1 and β-catenin correlated with poor prognosis.

Yasusei Kudo *et al* (2004)[30] isolated highly invasive clones from an OSCC cell line established from a lymph node metastasis by using an *in vitro* invasion assay method and compared the abnormalities of cell adhesion molecule E-cadherin and β-catenin in those cells. The isolated, highly invasive clones showed significant invasive capacity and reduction of E-cadherin and membranous β-catenin protein in comparison with parent cells. They found a reduced expression of E-cadherin which they attributed to methylation of its promoter region. They also found a reduced expression of membranous β-catenin in invasive and metastasis areas of OSCC. The reduced expression of membranous β-catenin was due to its protein degradation. They concluded that E-cadherin methylation and β-catenin degradation can be a novel target for inhibition of invasion and metastasis of OSCC as well as a marker for prediction of metastasis.

Soichi Iwai *et al* (2005)[31] studied the Mutations of the APC, β-catenin and axin 1 using single strand conformation polymorphism (SSCP) method and direct sequencing analysis and also studied the cytoplasmic accumulation of β-catenin by immunohistochemical staining and immunoblot analysis in the tissues and cell lines derived from oral squamous cell carcinoma. They found mutation in the APC

and Axin 1 gene without amino acid substitution and also cytoplasmic accumulation of β-catenin in 75% of cell lines and 90% of cancer tissue samples. They concluded that Axin 1 gene may be a mutational target in oral squamous cell carcinoma and cytoplasmic β-catenin accumulation is a characteristic feature but not associated with mutation of the APC and β-catenin genes.

F. Mahomed *et al* (2006)[32] studied the expression of E-cadherin and β-catenin in oral squamous carcinoma with and without nodal metastasis using immunohistochemistry. They found a significant association between E-cadherin / β-catenin and tumor differentiation but loss of expression in invasive tumor front in 93% of cases for E-cadherin and 73% of cases for β-catenin irrespective of the nodal status. They concluded that there is an association between loss of expression of E-cadherin / β-catenin and a lower degree of differentiation; and their use as markers of nodal metastasis in oral squamous carcinoma appears unreliable.

Lihong Wand *et al* (2007)[33] studied the simultaneous expression of desmoglein 3, desmocollin 3 and β-catenin in oral squamous cell carcinoma using immunohistochemistry and tried to correlate it with lymph node metastasis and cell proliferation. They found a reduced or loss of expression of all markers compared to normal oral epithelium. Also found a reduced or loss of expression of Dsc3 in advancing histological grade (moderately or poorly differentiated), and

reduced or loss of expression of β-catenin in lymph node metastasis. A positive correlation was found between reduced or loss of β-catenin and Dsc3 staining in lymph node metastatic cancer tissue. They concluded that abnormal expression of Dsc3, Dsg3, and β-catenin serve as a prognostic marker for oral squamous cell carcinoma.

CAI Zhi-gang *et al* (2008)[22] studied the expression pattern of β-catenin in oral squamous cell carcinoma using immuno-histochemistry. They found a reduced expression of β-catenin in cell membrane and increased expression in cytoplasm and nucleus in higher grade of malignancy. They concluded that a reduced membranous expression of β-catenin is associated with lymph node metastasis and it could be used as a prognostic marker to assess the biological behivour of cancer progression .

Soichi Iwai *et al* (2010)[34] studied the involvement of Wnt- β-catenin pathway in invasion and metastasis of oral squamous cell carcinoma cells. They transfected a mutated ß-catenin cDNA that lacks the entire exon-3 including phosphorylation sites specific to glycogen synthesis kinase-3ß into Ca9-22 cells. They found an increased expression of transcription factor T-cell factor (Tcf) / lymphoid enhancer factor (Lef)-dependent reporter gene activity as well as up-regulation of Wnt / ß-catenin target gene matrix metalloproteinase (MMP)-7 in these mutated cells. They also found redistribution of E-cadherin, rearrangement of actin filaments, and the elevation of

active Rho family members, Cdc42 and Rac. They concluded that β-catenin has an important role in invasion and metastasis of oral squamous cell carcinoma.

Lai-Kui Liu *et al* (2010)[35] studied the immunohistochemical expression of vimentin, E- cadherin and β-catenin complex in oral squamous cell carcinoma. They found an increased expression of vimentin and decresed expression of E-cadherin in recurrent tumors. Decresed β-catenin expression was seen in invasive tumor front. They concluded that vimentin, E- cadherin and β-catenin may serve as a useful prognostic marker in oral squamous cell carcinoma.

S.Y. Chaw *et al* (2012)[5] studied the role of E-cadherin, β-catenin, APC and vimentin as epithelial to mesenchymal transition biomarkers in oral squamous cell carcinoma using using a novel organotypic cell invasion model based on human dermis. They found a decreased E- cadherin expression and an increased vimentin and APC expression with increased disease severity. A shift in β-catenin expression from membranous to cytoplasmic / nuclear staining was noticed with increase in histopathological grade of severity. They concluded that aberrant expression of β-catenin, APC and Vimentin are potential markers of malignant transformation.

Khaled Waleed Zaid *et al* (2014)[9] performed an immunohistochemical assessment of E-cadherin and β-catenin in different histological grades of Oral Squamous Cell Carcinoma. They found a significant decrease in E-cadherin expression as the grade advanced. A significant correlation was found between β-catenin expression and histological grades. Expression of β-catenin shifted from membrane to cytoplasm and nuclear as a histological grades advanced. They also found a significant correlation between E-cadherin and β-catenin expression. Thus the can be used as prognostic marker in oral squamous cell carcinoma.

p63

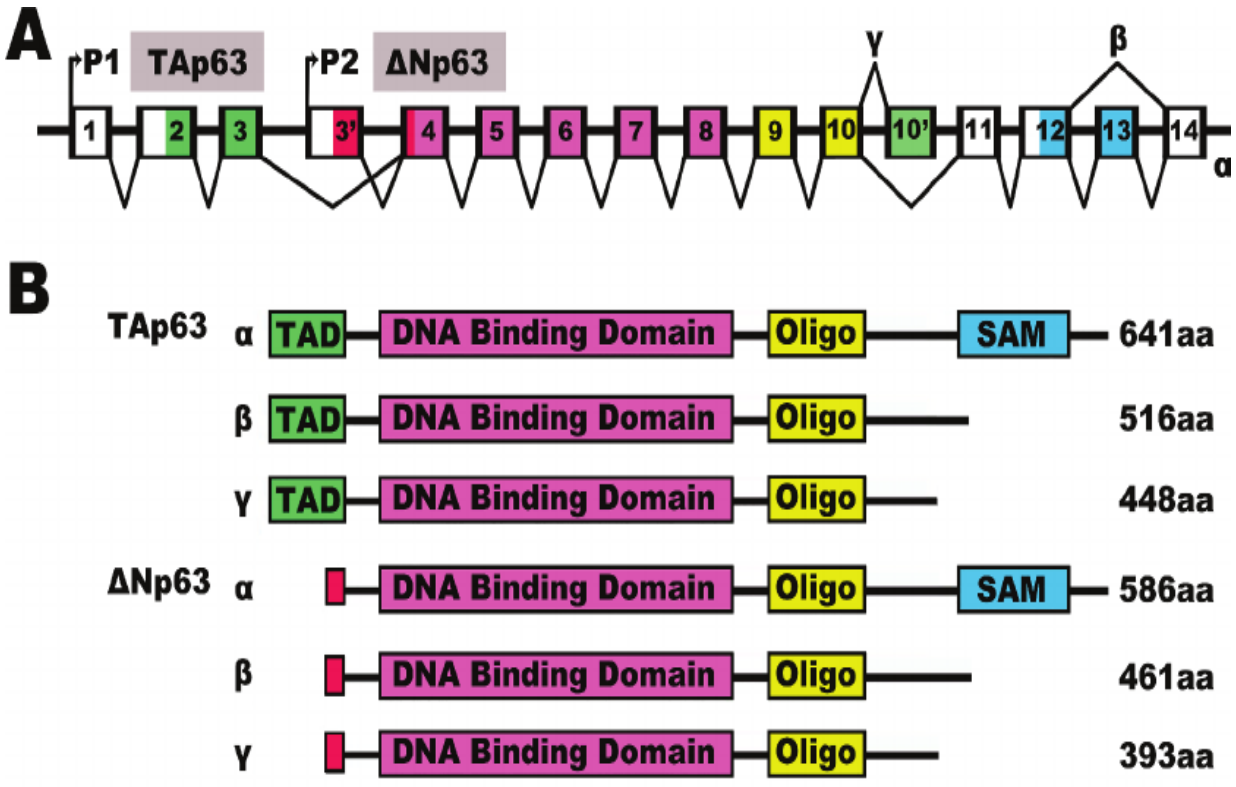
P53 is a tumor suppressor gene first described in 1979. Two homologue of p53 namely p63 and p73, which has structural similarity to p53 were discovered in 1997[19]. The p53 family consist of 3 main domains which are N-terminal transactivation domain (TAD), DNA binding domain (DBD) and the oligomerization domain (OD). Human p63 gene contains 14 exons spreading more than 250kb on chromosome 3q27[19,20].

Alternative splicing at 3' end of RNA leads to the formation of three p63 isoforms namely α, β and γ which differs in C- Terminal length. Based on the presence of transactivating (TA) domain in the N- Terminal two different isoforms are produced. Those with the TA domain are the TAp63 isoforms and those which lack the TA domain are the truncated isoforms ΔNp63. Thus in total six different isoforms of p63 are present which are TAp63α, TAp63β, TAp63γ, ΔNp63α, ΔNp63β, ΔNp63γ[19,20]. (Figure 2)

The TAp63 isoforms transactivates by binding to p53 responsive reporter genes, thus functions similar to p53. On the other hand the ΔNp63 isoforms which lacks TA domain competitively binds to p53 consensus sites through DNA binding domain, thus acting as dominant negative inhibitor of p53 and p63 transactivation[19,20].

P63 plays an important role in the maintenance of surface epithelium. It maintains the stem cell population and also plays an important role in differentiation of squamous epithelium. Thus any alteration in p63 expression could be a contributing factor to neoplastic cell transformation.

Figure 2: The human p63 splicing isoform. (A) Schematic representation of intron/exon structure of the human p63 gene. (B) Schematic representation of the human p63 protein splicing variants.



p63

Karin Nylander *et al* (2000) [36] studied the expression pattern of p63α and ΔNp63α in benign and malignant oral epithelial lesion using immunohistochemistry. They found that p63 isoforms were expressed in the nucleus of many cells. In normal and benign lesions, p63α/ΔNp63α-expressing cells were mainly found suprabasally, whereas p53-expressing cells were restricted to the basal-cell layer. With RT-PCR, they found ΔNp63α is the predominant isoform in cell lines from squamous-cell carcinomas of the head and neck. They concluded that p63 isoforms has a distinct role in growth and differentiation of epithelial cells and is highly amplified in different grades of squamous cell carcinoma.

Hai Hu *et al* (2002)[37] investigated the expression of p63 protein in human esophageal squamous cell carcinomas using immunohistochemistry and reverse transcriptase polymerase chain reaction. They found that the ΔNp63 mRNA was easily detectable in all malignant and histologically normal tissues, whereas TAp63 presented extremely low or no expression. The expression was found in the basal and Suprabasal layer. They concluded that increased p63 expression could be an early marker in esophageal squamous cell carcinomas.

Hong-Ran *et al* (2002)[38] studied the differential expression of p53 gene family members p63 and p73 in HNSCC by immunohistochemical analysis and compared it with clinicopathologic

parameters in 38 patients. They found that in histologically normal epithelium p53 and p73 showed basal and/or parabasal expression. p53 expression was weak and discontinuous while a stronger expression of p63 in suprabasal cells was found. In dysplasias, all three markers showed a gradual increase in the extent and intensity of cellular expression with histologic progression. In carcinomas, p63 was highly expressed followed by p73 and p53. Significant correlation was found between p63 and p73 expression. Distant metastasis and perineural/vascular invasion showed p73 expression. They concluded that p63 and p73 expression is associated with head and neck cancer development and progression.

Karin Nylander *et al* (2002)[39] studied the differential expression of p63 isoforms in normal tissues and neoplastic cells by producing three antibodies produced against the two N-terminal isoforms (TAp63 and Np63) and the C-terminal region of the p63*α* proteins. TAp63 proteins were located suprabasally in stratified epithelia compared with the N-terminal truncated forms, which were more abundantly expressed in the basal cell layer, indicating a switch in expression of p63 isoforms during normal cellular differentiation. In squamous cell carcinomas Np63*α* was the widely expressed isoform, thus a role in promoting neoplastic cell growth. TAp63, but not Np63 or p63*α*, was detected in normal colon and in colon carcinoma. TAp63 proteins were also expressed in the nuclei of a sub-population of lymphoid cells and in most malignant lymphomas, whereas Np63

proteins were not expressed. They concluded that different p63 proteins are expressed during differentiation and progression of neoplasia.

Keith E. Matheny *et al* (2003)[40] studied the molecular relationship between epidermal Growth Factor Receptor (EGFR) and p63 expression using ZD1839, an adenosine triphosphate competitive inhibitor specific to the EGFR tyrosine kinase, in human head and neck squamous cell carcinoma cell line, SCC-012 using flow cytometry. p63 protein and mRNA levels were analyzed by Western and Northern blot analyses. They found a dose-dependent decrease in p63 protein and mRNA levels over the course of ZD1839 treatment. Levels of phosphorylated MAPK decreased and p27KIP-1 levels increased after ZD1839 treatment. ZD1839 treatment induced a twofold increase in G1-phase cells and a 3.5- fold decrease in S-phase cells consistent with growth arrest. They conclude that p63 is a downstream target of EGFR signaling.

Paul C. Edwards *et al* (2004)[41] studied the immunohistochemical expression of p63 in benign (basal cell and canalicular adenomas) and malignant (adenoid cystic carcinoma (ACC), polymorphous low-grade adenocarcinoma (PLGA)) salivary gland neoplasms. PLGA showed 100% positivity and ACC showed 87% of positivity primarily in the nonluminal myoepithelial-like cells. Canalicular adenoma showed no immunoreactivity. All basal cell

adenomas of parotid origin stained strongly for p63 but those from upper lip showed no staining. They concluded that p63 is not an ideal marker to distinguish between ACC, PLGA, and basal cell adenoma.

Maria P. Foschini *et al* (2004)[42] performed an immunohistochemical study to define the role of p63 in oral squamous cell carcinomas using a monoclonal antibody recognising all p63 isoforms and an anti-Ki67 antibody. p63 mRNA expression pattern was evaluated using reverse-transcription polymerase chain reaction (PCR) and nested PCR. They found that p63 was positive in all OSCC similar to ki67. The percentage of p63 positive cells increased from normal to neoplastic mucosa. However there was no relationship between the number of p63 positive cells and prognosis. In metastases the truncated isoforms D4TAp63 and DNp73L were more frequently expressed. They concluded that impaired p63 isoform expression have a role in cell proliferation and indirectly enhance the metastasising capacity of OSCC.

Marcelo C. Bortoluzzi *et al* (2004)[43] assessed the p63 expression in oral squamous cell carcinomas and dysplasias using immunohistochemistry. They also assessed p53 and ki67 in this lesions. They found that p63 stained suprabasally in the entire non invasive lesion, most nuclei in invasive lesion stained positive for p63, moderately differentiated SCC stained more than that of well differentiated SCC. They concluded that though p63 staining was

evident in all lesions it could not be used to differentiate the lesion. p63 is not coexpressed with p53 expression or Ki-67 suggesting functional independence.

Joseph C. Sniezek *et al* (2004)[44] studied the differential expression of p63 isoforms in head and neck squamous cell carcinoma and oral lichen planus using immunohistochemistry. Western blot analysis was done to confirm the p63 isoforms. To analyse and quantitatively compare p63 isoform expression at the RNA level RT-PCR was performed. They found p63 expression in all tumors and normal tissue specimens and found ΔNp63α to be the major isoform expressed. They also found underexpression of p63 in oral lichen planus. They concluded that ΔNp63α plays antidifferentiation and anti-apoptotic role in HNSCC.

Niklas Thurfjell *et al* (2004)[45] performed real time RT-PCR to study p63 isoform HNSCC. They found ΔNp63α to be the major isoforms and that it is highly expressed in tumors compared to normal tissue. They found that p63β was also highly expressed in tumors. However correlations between different p63-isoform expression patterns and proliferation, p53 status, or telomerase expression could not be found. The high expression of ΔNp63 found in basal layers of normal epithelium were similar to those found in tumors. They suggested that high-level expression of ΔNp63 in tumour cells represents the maintained expression by the basal cells from which the

tumour originated, rather than representing a true over-expression of p63 during tumourigenesis. They also showed that tobacco usage had no effect on p63 expression in oral epithelium.

James W. Rocco *et al* (2006)[46] demonstrated the role of ΔNp63α as an essential survival factor in HNSCC. They studied the p63 isoform expression in human HNSCC derived cell lines JHU-029 expressing TAp63 and JHU-011 expressing truncated mutant p63. They found that p63 expression is three to five times higher than normal in HK cell lines. Through QRT-PCR ΔNp63 mRNA was found to be the predominant isoform. They demonstrated the functional role of endogenous p63 in HNSCC cells by RNA mediated interferences using p63 targeted small hairpin RNA(shRNA) and found that specific inhibition of p63 in significant fraction of cells undergoing apoptosis. With this they showed that p63 promotes survival of squamous cell carcinoma cells by suppressing the proapoptotic function of p73.

Lorenzo Lo Muzio *et al* (2005)[47] investigated the role p63 in oral cancer and its potential as prognostic marker using immunohistochemistry. p63 expression was seen in basal and parabasal layer in normal tissues, carcinoma in situ and well differentiated neoplasm. Diffuse staining was seen in grade II neoplasm. Completely dedifferentiated neoplasm showed negative or faint staining. Intense and diffuse labelling was seen in infiltrating neoplasm. No staining was observed in keratin pearl areas. They found no significant

correlation between p63 expression, sex, age, tumor size, staging, recurrence and metastasis. They concluded that p63 expression can be useful to identify aggressive and invasive OSCC and can be used as a prognostic marker.

Lucinei Roberto de Oliveira *et al* (2006)[48] investigated the impact of p53 and p63 immunoexpression in oral squamous cell carcinoma. P63 expression was found in the well and moderately differentiated tumors and absent in terminally differentiated cells and keratin pearl areas. P53 was found in 52% of tumors. No significant correlation was found between p63 expression and both tumour recurrence and metastasis. p53 positive cases showed higher metastatic rate. They conclude that p53 over-expression and decreased intensity of p63 staining is associated with metastasis.

Anju Sinha *et al* (2015)[49] studied the expression of p63 in potentially malignant and malignant lesions using immunohistochemistry. Increased expression and mean labeling index of p63 was seen in oral sub mucous fibrosis (57%), epithelial dysplasia (63%) and carcinoma (70%). They suggested that increased labelling index and supra basal expression of p63 in dysplasia can be used as a marker for pre-malignancy.

Nasrollah Saghravanian *et al* (2017)[50] studied the correlation of p63 and CD44 expression with clinicopathological parameters like histological grading, TNM staging, overall survival rate, patient age, gender and tumor location. No significant relation found between the markers and patients age, gender, tumor location and overall survival rate. Increased CD44 and p63 expression was seen in higher grades of carcinoma. They suggested that these markers can be further studied to understand the pathogenesis of OSCC and a potential target in cancer treatment.

MATERIALS AND METHODS

STUDY DESIGN AND CASE SELECTION:

This immunohistochemical study, to analyse the immunohistochemical expression of p63 and β-catenin was done on the archival retrieved formalin fixed, paraffin embedded tissues obtained from the Department of Oral Pathology, Adhiparasakthi Dental College and Hospital, Melmaruvathur.

The study group includes histologically diagnosed

- Well differentiated squamous cell carcinoma- 10
- Moderately differentiated squamous cell carcinoma- 11
- Normal tissue for control- 15
- Total no of samples- 36
- Control group includes biopsies from the normal buccal mucosa or gingiva adjacent to the site of surgery during the surgical removal of third molar in 15 patients.

For positive control, archival retrieved formalin fixed, paraffin embedded colon cancer for β-catenin staining and normal prostate for p63 staining was obtained from the Department of General Pathology, Melmaruvathur Adhiparasakthi Institute of Medical Science and Research, Melmaruvathur.

ARMAMENTARIUM[14,15,22] **(Figure-3)**

- Microtome (Thermo scientific, MICROM HM340E)
- Paint brush
- Disposable microtome blades
- Hot plate
- Hot water bath
- PathnSitu positively charged slides
- Pressure cooker (5 Liters)
- Measuring Jars
- Coplin Jars
- Electronic Timer
- Absorbent wipes
- Coverslip for slides
- Binocular Light Microscope (Olympus CX21i)
- Micropipette
- Rectangular steel trough
- Induction stove
- Incubator (Hitech Equipments)
- Liquid repellent slide marking pen
- Deparaffinization stainless steel staining trough and rack
- pH meter (E1 digital pH meter)
- A DELTA PLAN2 AP40 Trinocular Light Microscope with camera Head

- **ANTIBODIES:**

1.**Primary antibody**

a) Anti-p63 [Mouse monoclonal antibody] – 4A4 (PathnSitu Biotechnologies Private Limited)

b) Anti-βcatenin [Rabbit monoclonal antibody] - EP35 (PathnSitu Biotechnologies Private Limited)

2. **Secondary kit** (PolyExcel HRP/DAB Detection System) - PathnSitu Biotechnologies Private Limited

a) PolyExcel H_2O_2

b) PolyExcel Target Binder

c) PolyExcel Poly HRP

d) PolyExcel stun DAB – Chromogen

e) PolyExcel stun DAB – Buffer

- **REAGENTS**[14,15,22]**:**
  - Tris-EDTA Buffer – 50X concentration (PathnSitu Biotechnologies Private Limited)
  - Immuno wash Buffer – 25X concentration (PathnSitu Biotechnologies Private Limited)
  - Distilled water
  - Xylene
  - Absolute alcohol (Isopropyl Alcohol)
  - Alcohol 90% (Isopropyl Alcohol)
  - Alcohol 70% (Isopropyl Alcohol)

- Harris Hematoxylin
- Mountant (Dibutyl Phthalate Xylene)

IHC METHODOLOGY[14,15]:

- Formalin fixed paraffin embedded tissues were sectioned at 3µm and mounted on charged slides and kept for overnight incubation at 37^{o}C
- Prior to staining slides were incubated at 60 – 70 ^{o}C for 1 hour
- Deparaffinized by 2 changes of xylene 10 minutes each
- Hydrated through descending grades of alcohols as follows:
  - Absolute alcohol – 1 change, 5 minutes
  - 90% alcohol – 5 minutes
  - 70% alcohol – 5 minutes
- Distilled water wash 2 changes each for 2 minutes
- Antigen retrieval done for 15- 20 minutes (upto 2 whistles in pressure cooker)
- Cooled for minimum of 30 minutes
- Distilled water wash 2 changes each for 2 minutes
- Washed in PBS / TBS for 2 minutes
- Circles were marked enclosing the section using liquid repellent pen
- Endogenous peroxidase blocking was done by adding PolyExcel H_2O_2 on the section, keep for 5 minutes
- Washed in wash buffer for 5 minutes, 3 changes

- Primary antibody was added and kept for 30 minutes for p63 and 45 minutes for β catenin in a moist chamber
- Washed in wash buffer for 5 minutes, 3 changes
- PolyExcel Target Binder reagent was added and incubated for 12 minutes
- Washed in wash buffer for 5 minutes, 3 changes
- Polyexcel HRP was added and incubated for 12 minutes
- DAB solution was prepared (1 ml of DAB buffer + 1 drop DAB chromogen)
- Washed in wash buffer for 5 minutes, 3 changes
- Working DAB chromogen was added and kept for 2-5 minutes, then washed in distilled water.
- Counterstained with hematoxylin for 30 seconds
- Washed in running tap water for 5 minutes
- Dehydrated through successive changes of alcohol and clear with xylene
- Dried and mounted with DPX

POSITIVE CONTROLS

1. Positive control section for p63 includes normal prostate and was treated in the same manner as the test groups.
2. Positive control section for βcatenin includes colon cancer and was treated in the same manner as the test groups.

NEGATIVE CONTROLS

One section of test sample was selected and treated in the same manner as the test groups except that, the primary antibody was omitted for both p63 and βcatenin.

ANALYSIS OF IMMUNOREACTIVITY OF p63 AND β-CATENIN :

To know the expression pattern and also to determine the levels of protein expression in the epithelial layers, area of staining was analysed. It was determined by scanning the entire section of the epithelium on the surface and also the invaded cells and islands in the case of carcinoma and was recorded as

SCORE	INFERENCES
0	0%
1	<25%
2	25 - 49%
3	50 - 74%
4	75 - 100%

To know the extent of stain uptake, intensity of staining was analysed. Ten random fields were selected at 40x magnifications and were scored as:

SCORE	INFERENCES
0	No stain
1	Mild staining
2	Moderate staining
3	Intense staining

Figure 3 : ARMAMENTARIUM

Fig 3 (a) Microtome

Fig 3 (b) Induction stove and Pressure cooker

Fig 3 (c) Electronic Timer

Fig 3 (d) Microscope

Fig 3 (e) Micropipette

Fig 3 (f) Incubator

Fig 3 (g) Reagent blocker

Fig 3 (h) Deparaffinization stainless steel staining trough and rack

Fig 3 (i) Primary antibody Anti - p63[Mouse Monoclonal antibody]

Fig 3 (j) Primary antibody Anti– βcatenin [Rabbit Monoclonal antibody]

Figure 3 (k) Secondary kit

Figure 3(l) DAB Chromogen and DAB buffer[H2O2, Target Binder, Poly HRP]

Fig 3 (m) Tris – EDTA

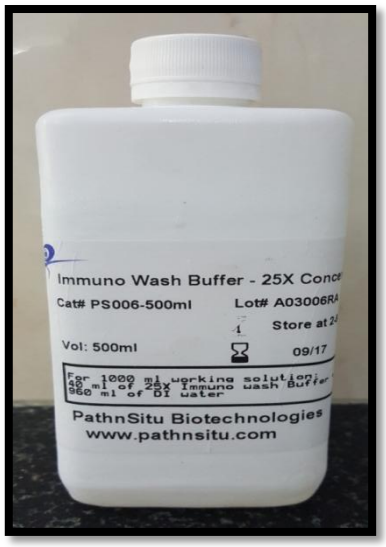


Fig 3 (n) Wash Buffer

Fig 3 (o) Hematoxyline

RESULTS

The present study was undertaken to analyse the immunohistochemical expression of p63 and β-catenin in different grades of oral squamous cell carcinoma. Previously diagnosed 10 cases of well differentiated squamous cell carcinoma, 11 cases of moderately differentiated squamous cell carcinoma and 15 normal tissues as control were selected from the departmental archives.

Serial sections of 3μ thickness were made and stained separately for p63 and β-catenin using immunohistochemistry. Intensity of staining was assessed by selecting 10 random fields under high power magnification and scored as 0- no stain, 1-mild, 2-moderate, 3-Intense. The area of staining was determined by scanning the entire section for epithelial uptake of stain and scored as 0(0%), 1(<25%), 2(25-49%), 3(50 - 74%), 4(75 - 100%).

Scoring was done for p63 (n=36) and β-catenin (n=36) [Annexure 2]. The staining intensity scores for β-catenin ranged between 1 & 3, 0 & 3 for well differentiated and moderately differentiated carcinoma respectively. The normal tissues uniformly scored 3.

The staining intensity scores for p63 ranged between 2 & 3, 1 & 3 for normal and moderately differentiated carcinoma respectively. The well differentiated carcinoma uniformly scored 3.

The scores for β-catenin area of staining ranged between 3 & 4, 1&4,0 &4 for normal, well differentiated and moderately differentiated carcinoma respectively.

The scores for p63 area of staining ranged between 2 & 4 for both normal and well differentiated carcinoma. The score ranged between 1 &4 for moderately differentiated carcinoma.

The data obtained was statistically analysed.

β-CATENIN

Table 1: Comparison of *β*-catenin intensity and area of staining between the groups

β-catenin		N	Mean	Std. Deviation	Minimum	Maximum	P-value
Staining intensity	Normal	15	3.00	0.000	3	3	0.001*
	Well Differentiated Carcinoma	10	2.10	0.876	1	3	
	Moderately Differentiated carcinoma	11	1.82	1.168	0	3	
Area	Normal	15	3.80	0.414	3	4	0.002*
	Well Differentiated carcinoma	10	2.60	1.075	1	4	
	Moderately Differentiated carcinoma	11	2.45	1.293	0	4	

* denotes statistically significant using Kruskall Wallis' ANOVA

Intensity of Staining

The mean score for intensity of staining of normal tissue, well differentiated carcinoma and moderately differentiated carcinoma was found to be 3.0 (SD 0), 2.1 (SD 0.87) & 1.82 (SD 1.16) respectively. The difference between the mean scores was found to be statistically significant ($p < 0.05$) using Kruskall Wallis ANOVA (Table 1).

Area of staining

The mean score for area of staining of normal tissue, well differentiated carcinoma and moderately differentiated carcinoma was found to be 3.80 (SD 0.4), 2.60 (SD 1.0) & 2.45 (SD 1.2) respectively. The difference between the mean scores was found to be statistically significant ($p < 0.05$) using Kruskall Wallis ANOVA (Table 1).

Table 2: Inter group comparison of β-catenin staining intensity using Mann Whitney U test

Groups	N	Mean	Std. Deviation	Minimum	Maximum	P-value
Normal	15	3.00	0.000	3	3	0.012*
Well Differentiated carcinoma	10	2.10	0.876	1	3	
Normal	15	3.00	0.000	3	3	0.005*
Moderately Differentiated carcinoma	11	1.82	1.168	0	3	
Well Differentiated carcinoma	10	2.10	0.876	1	3	0.654
Moderately Differentiated carcinoma	11	1.82	1.168	0	3	

* denotes statistically significant

We compared the staining intensity mean score between the normal and well differentiated carcinoma using Mann whitney U test and found the difference to be statistically significant (Table 2). That is there is decrease in intensity of β-catenin expression in well differentiated carcinoma compared to the normal tissues.

We compared the staining intensity mean score between the normal and moderately differentiated carcinoma using Mann whitney U test and found the difference to be statistically significant (Table 2). That is there is decrease in intensity of β-catenin expression in moderately differentiated carcinoma compared to the normal tissues.

We compared the staining intensity mean score between well differentiated and moderately using Mann whitney U test. However we did not find any significant difference between the mean scores (Table 2). That is there is not much difference in the intensity of β-catenin expression in well differentiated and moderately differentiated carcinoma.

Table 3: Inter group comparison of Area of β-catenin staining using Mann Whitney U test

Groups	N	Mean	Std. Deviation	Minimum	Maximum	P-value
Normal	15	3.80	0.414	3	4	0.004*
Well Differentiated carcinoma	10	2.60	1.075	1	4	
Normal	15	3.80	0.414	3	4	0.005*
Moderately Differentiated carcinoma	11	2.45	1.293	0	4	
Well Differentiated carcinoma	10	2.60	1.075	1	4	0.863
Moderately Differentiated carcinoma	11	2.45	1.293	0	4	

* denotes statistically significant

We compared the mean scores of area of staining between the normal and well differentiated carcinoma using Mann whitney U test and found the difference to be statistically significant (Table 3). That is, there is decrease in area of β-catenin expression in well differentiated carcinoma compared to the normal tissues.

We compared the mean scores of area of staining between the normal and moderately differentiated carcinoma using Mann whitney U test and found the difference to be statistically significant (Table 3). That is, there is decrease in area of β-catenin expression in moderately differentiated carcinoma compared to the normal tissues.

Next we compared the mean scores of area of staining between the well differentiated and moderately differentiated carcinoma using Mann whitney U test and found that there is no significant difference in the scores obtained (Table 3). That is area of staining cannot be used to differentiate between well differentiated and moderately differentiated carcinoma.

p63

Table 4 : Comparison of p63 intensity and area of staining between the groups using Kruskall Wallis ANOVA

p63		N	Mean	Std. Deviation	Minimum	Maximum	P-value
Staining intensity	Normal	15	2.60	0.507	2	3	0.091
	Well Differentiated carcinoma	10	3.00	0.000	3	3	
	Moderately Differentiated carcinoma	11	2.64	0.674	1	3	
Area	Normal	15	3.20	0.775	2	4	0.439
	Well Differentiated carcinoma	10	2.80	0.632	2	4	
	Moderately Differentiated carcinoma	11	2.91	1.044	1	4	

Intensity of Staining

The mean score for intensity of staining of normal tissue, well differentiated carcinoma and moderately differentiated carcinoma was found to be 2.6 (SD 0.5), 3.0 (SD 0.0) & 2.64 (SD 0.6) respectively. The difference between the mean scores was not found to be statistically significant using Kruskall Wallis ANOVA (Table 4).

Area of staining

The mean score for area of staining of normal tissue, well differentiated carcinoma and moderately differentiated carcinoma was found to be 3.20 (SD 0.7), 2.80 (SD 1.6) & 2.91 (SD 1.0) respectively. The difference between the mean scores was found to be statistically not significant using Kruskall Wallis ANOVA (Table 4).

Table 5: Inter group comparison of p63 staining intensity using Mann Whitney U test

Groups	N	Mean	Std. Deviation	Minimum	Maximum	P-value
Normal	15	2.60	0.507	2	3	0.103
Well Differentiated carcinoma	10	3.00	0.000	3	3	
Normal	15	2.60	0.507	2	3	0.721
Moderately Differentiated carcinoma	11	2.64	0.674	1	3	
Well Differentiated carcinoma	10	3.00	0.000	3	3	0.314
Moderately Differentiated carcinoma	11	2.64	0.674	1	3	

* denotes statistically significant

We compared the p63 staining intensity mean score between the normal and well differentiated carcinoma using Mann whitney U test and found no significant difference. This shows that the staining intensity cannot be used to differentiate between normal and well differentiated carcinoma.

We compared the p63 staining intensity mean score between the normal and moderately differentiated carcinoma using Mann whitney U test and found no significant difference. This shows that the staining intensity cannot be used to differentiate between normal and moderately differentiated carcinoma.

We compared the p63 staining intensity mean score between the well differentiated and moderately differentiated carcinoma using Mann whitney U test and found no significant difference. This shows that the staining intensity cannot be used to differentiate between normal and moderately differentiated carcinoma.

Hence p63 staining intensity does not contribute to differentiate between normal and different grades of carcinoma.

Table 6: Inter group comparison of Area of p63 staining using Mann Whitney U test

Groups	N	Mean	Std. Deviation	Minimum	Maximum	P-value
Normal	15	3.20	0.775	2	4	0.216
Well Differentiated carcinoma	10	2.80	0.632	2	4	
Normal	15	3.20	0.775	2	4	0.54
Moderately Differentiated carcinoma	11	2.91	1.044	1	4	
Well Differentiated carcinoma	10	2.80	0.632	2	4	0.705
Moderately Differentiated carcinoma	11	2.91	1.044	1	4	

* denotes statistically significant

We compared the mean scores of p63 area of staining between the normal and well differentiated carcinoma using Mann whitney U test and found no significant difference (Table 6). This shows that area of p63 expression cannot be used to differentiate between the normal tissues and well differentiated carcinoma.

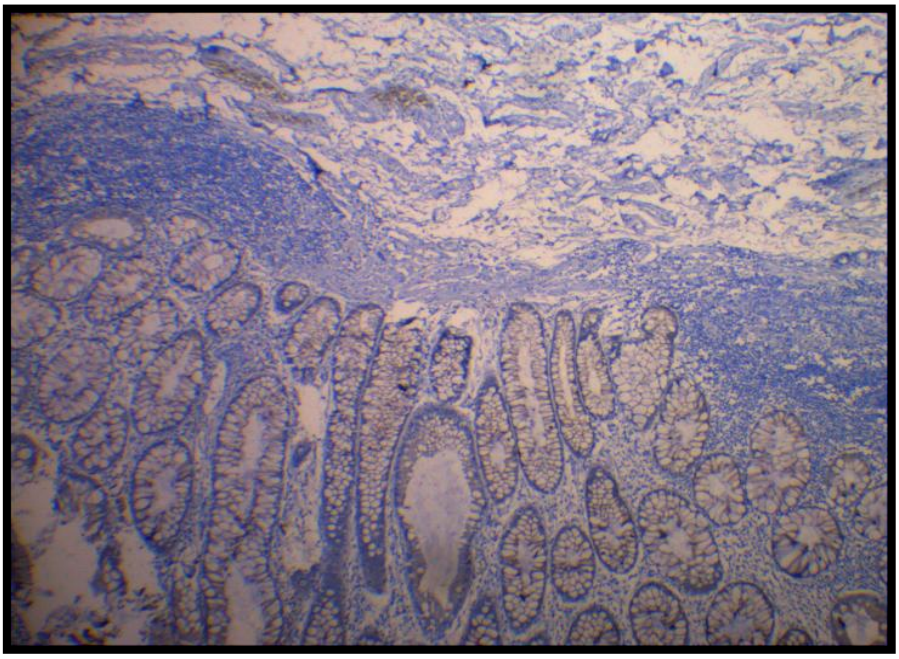
We compared the mean scores of p63 area of staining between the normal and moderately differentiated carcinoma using Mann whitney U test and found no significant difference (Table 6). This shows that area of p63 expression cannot be used to differentiate between the normal tissues and moderately differentiated carcinoma.

We compared the mean scores of p63 area of staining between the well differentiated and moderately differentiated carcinoma

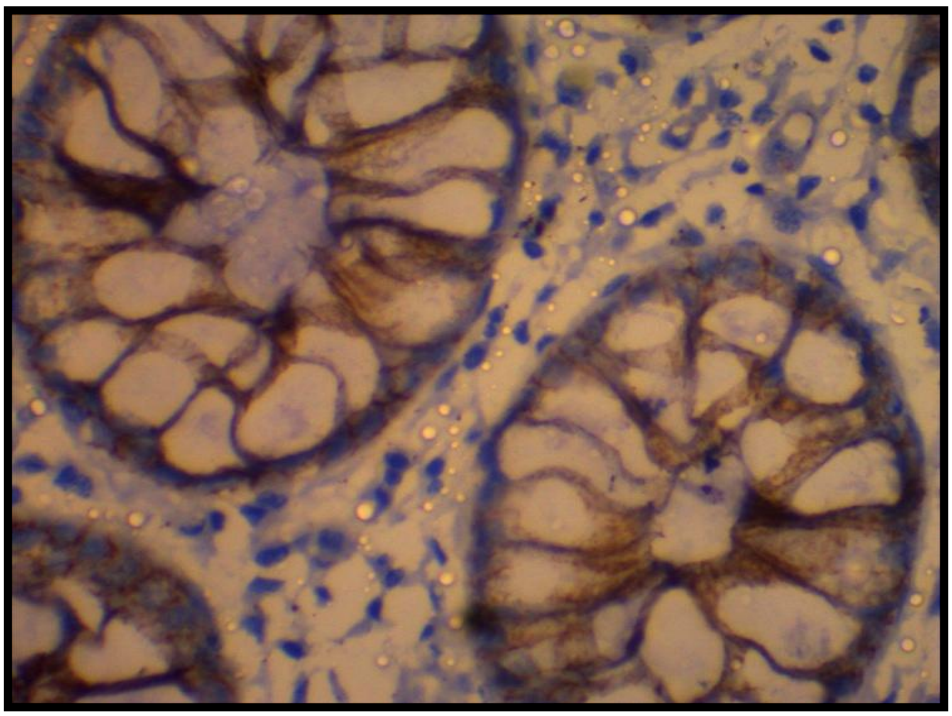
using Mann whitney U test and found no significant difference (Table 6). This shows that area of p63 expression cannot be used to differentiate between the well differentiated and moderately differentiated carcinoma.

Hence p63 area of staining does not play a significant role to differentiate between normal tissue and different grades of carcinoma.

Figure 4: Photomicrograph of IHC stained section showing strong positive cell membrane expression of β-catenin in colon cancer (positive control) at low power magnification(a) and at high power magnification(b).

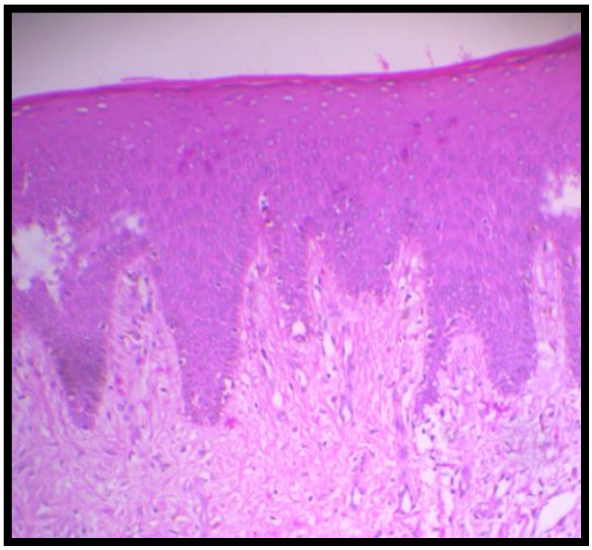


a)



b)

Figure 5 (a) : Photomicrograph showing H&E stained section in normal tissue at low power magnification. **(b & C):** Photomicrograph of IHC stained section showing membranous expression of β-catenin in normal tissue at low power magnification (b) & at high power magnification (c).



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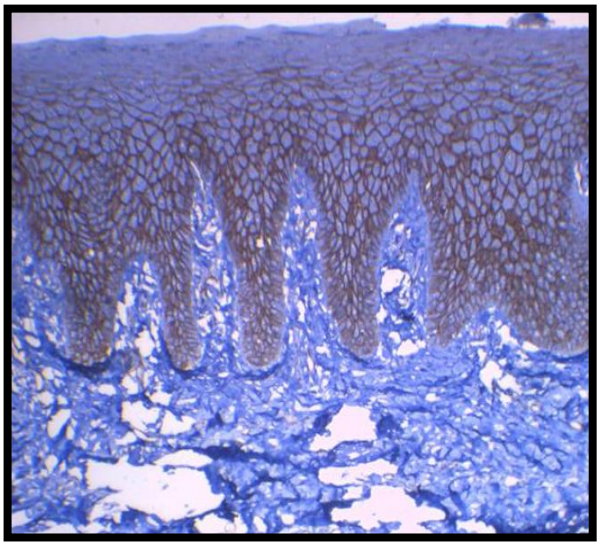

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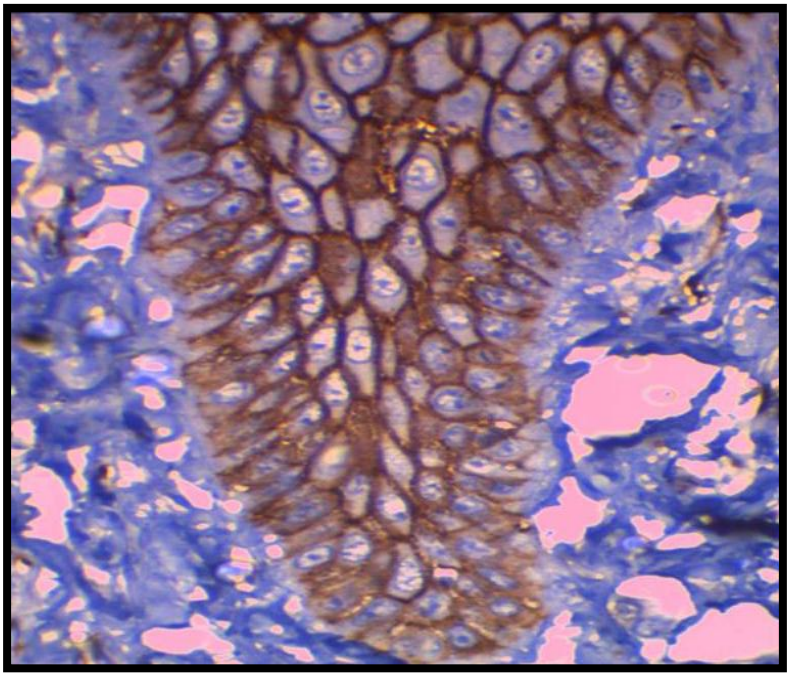
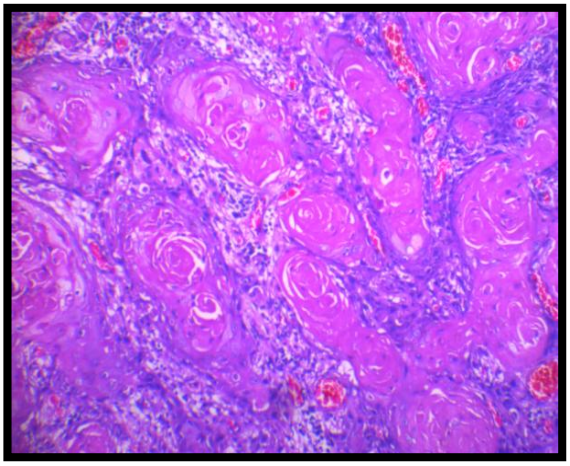
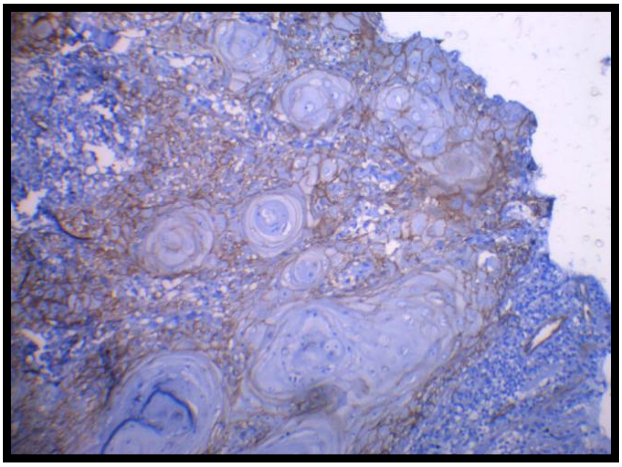


c)

Figure 6 (a): Photomicrograph showing H&E stained section in well differentiated SCC at low power magnification. **(b &c):** Photomicrograph of IHC stained section showing membranous expression of β-Catenin in well differentiated carcinoma at low power magnification (b) and at high power magnification (c).



a)



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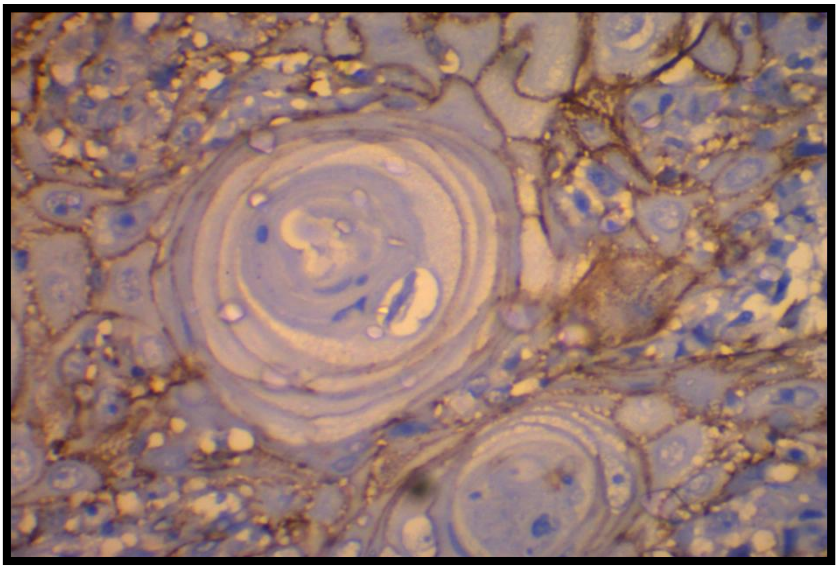
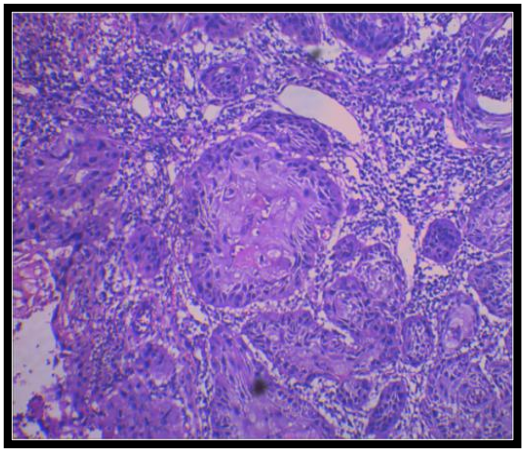


c)

Figure 7(a) : Photomicrograph showing H&E stained section of moderately differentiated carcinoma at low power magnification. **(b&c):** Photomicrograph of IHC stained section showing membranous and cytoplasmic expression of β catenin in moderately differentiated SCC at low power magnification (b) and high power magnification (c).



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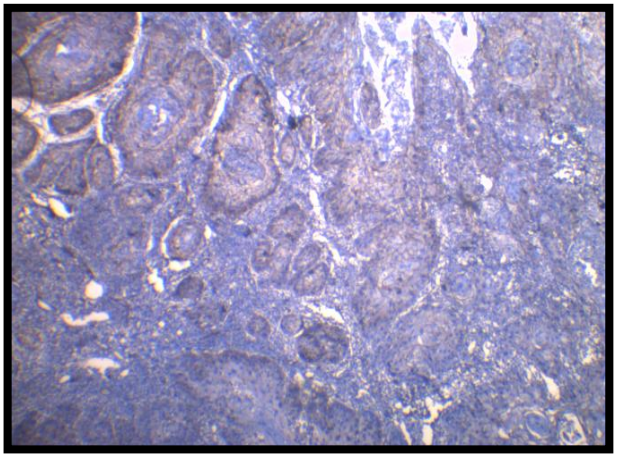

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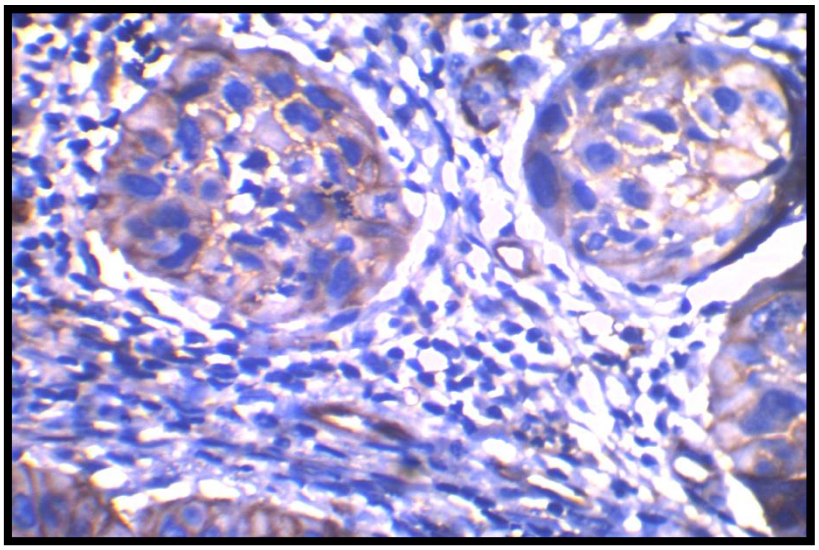
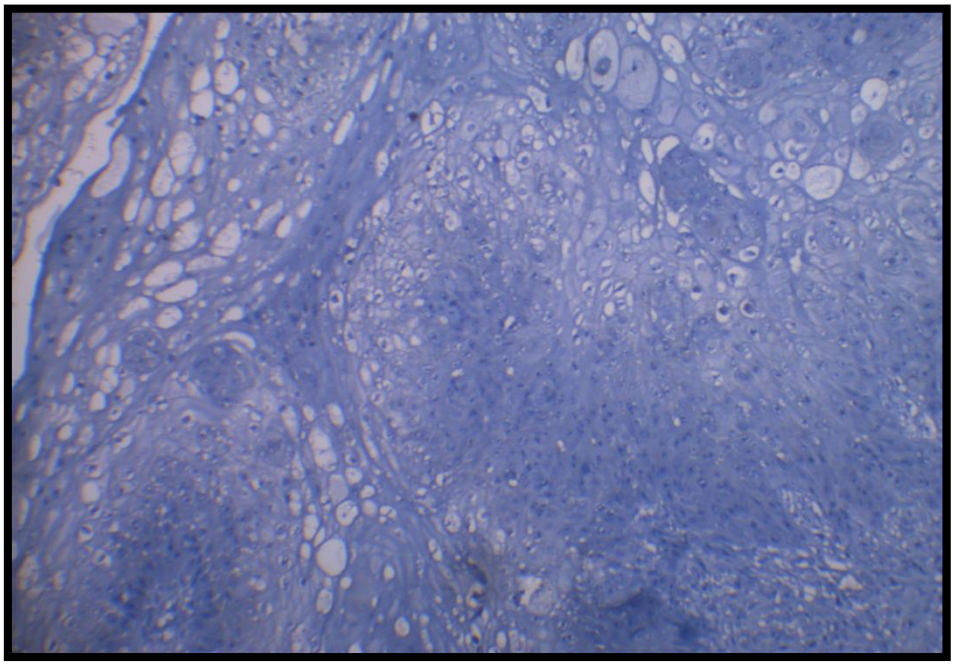


c)

Figure 8(a&b): Photomicrograph showing IHC stained section of moderately differentiated carcinoma showing no uptake of stain in invaded islands at low power magnification (a) and high power magnification(b)



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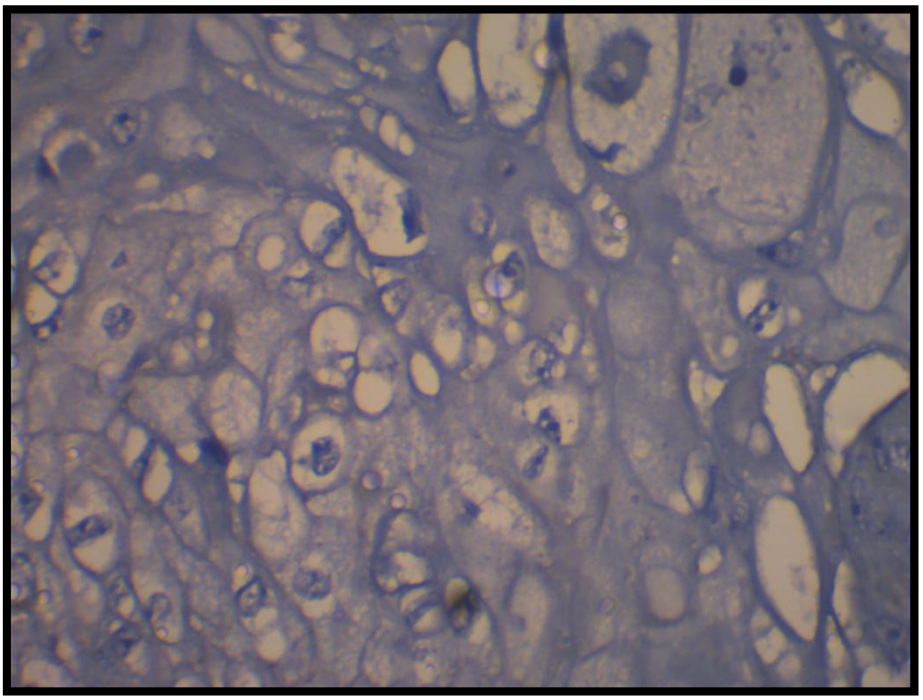
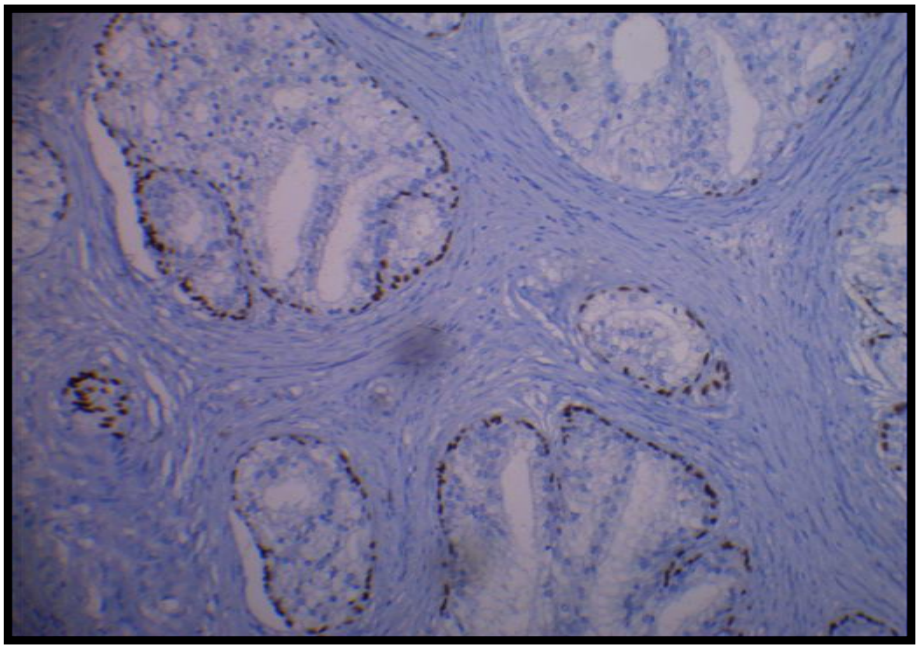
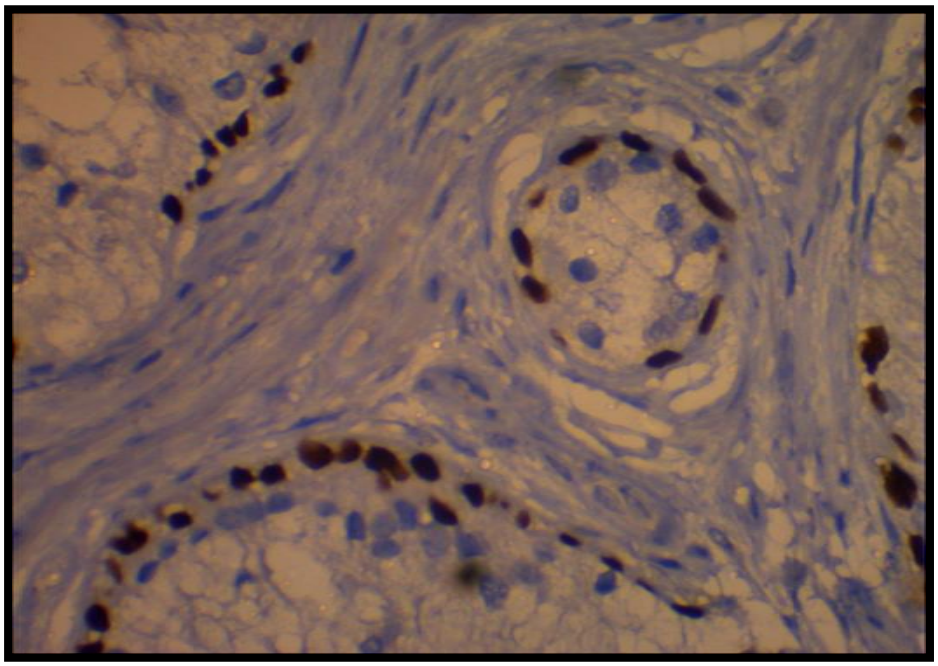


b)

Figure 9(a&b): Photomicrograph of IHC stained section showing strong positive nuclear expression of p63 in normal prostate (positive control) at low power magnification (a) and high power magnification (b)

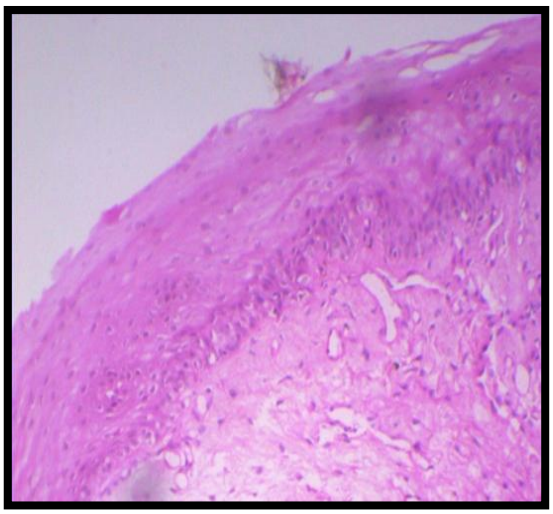


a)

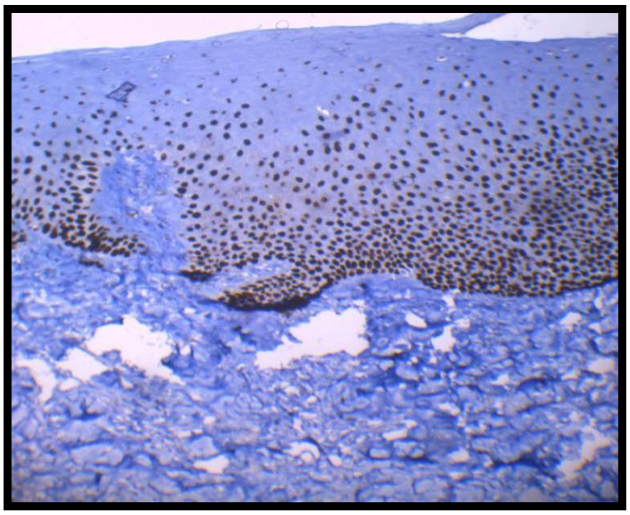


b)

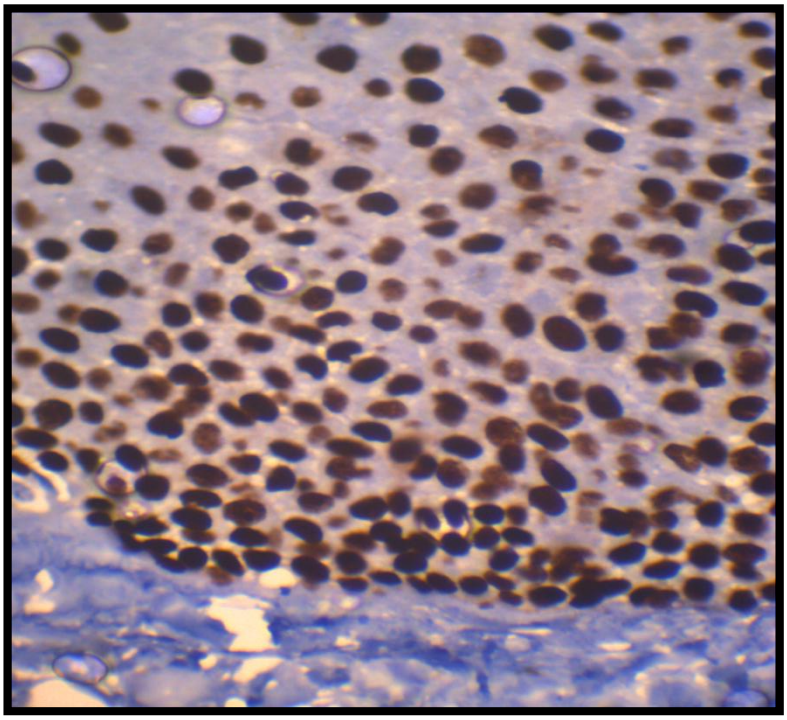
Figure 10(a): Photomicrograph showing H&E stained section showing normal tissue at low power magnification. **(b&c):** Photomicrograph of IHC stained section showing strong nuclear expression of p63 in normal tissue at low power magnification (b) and high power magnification (c) .



a)

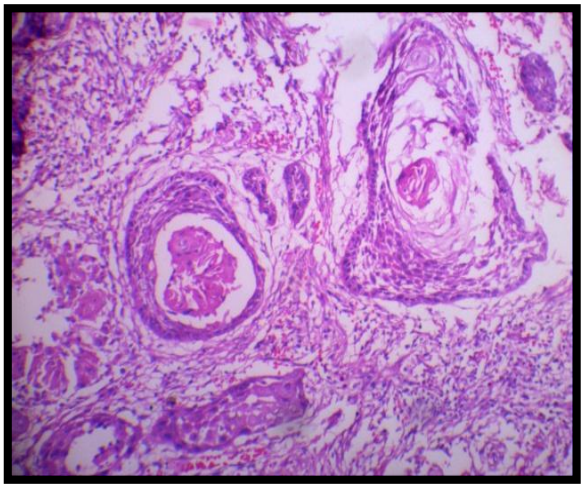


b)

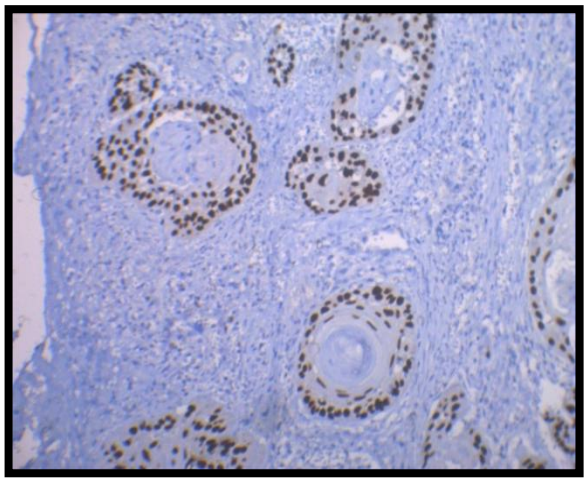


c)

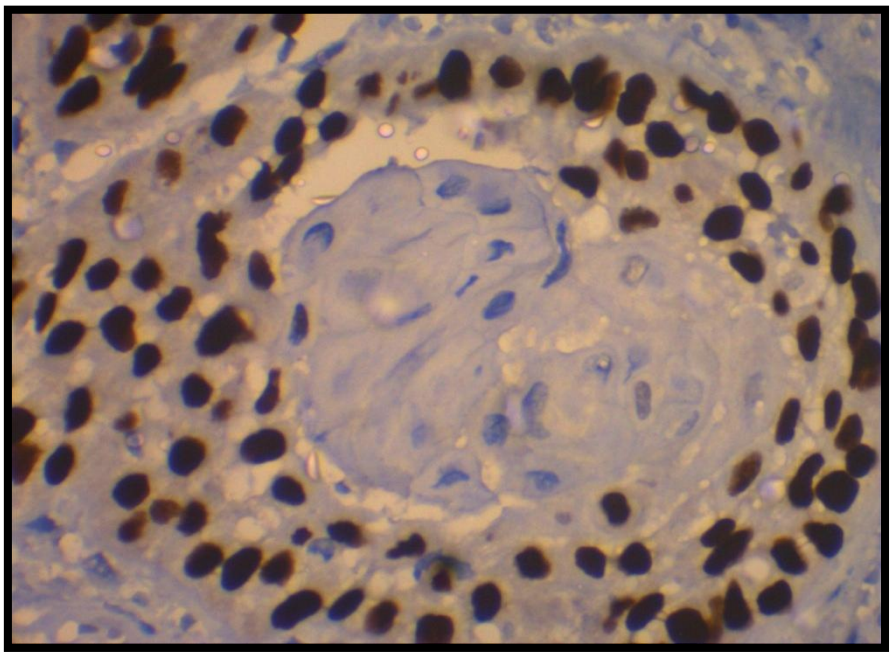
Figure:11(a) Photomicrograph showing H&E stained section well differentiated carcinoma at low power magnification. **(b&c):** Photomicrograph of IHC stained section showing strong nuclear expression of p63 in well differentiated carcinoma at low power magnification(b) and high power magnification (c).The lack of staining in the keratin pearls can be appreciated.



a)

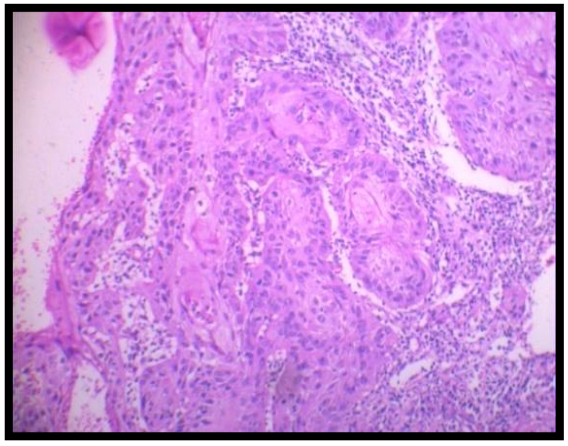


b)

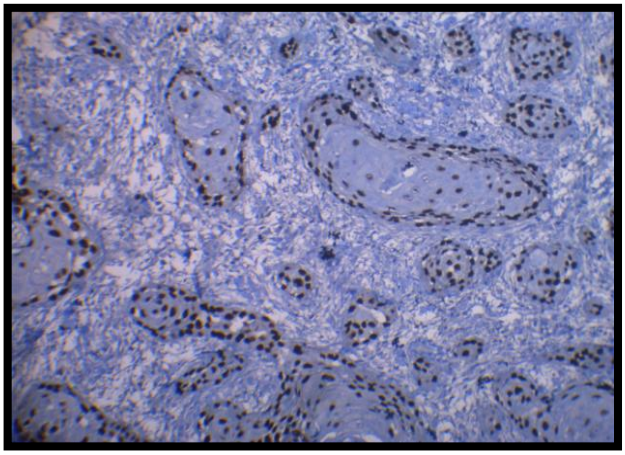


c)

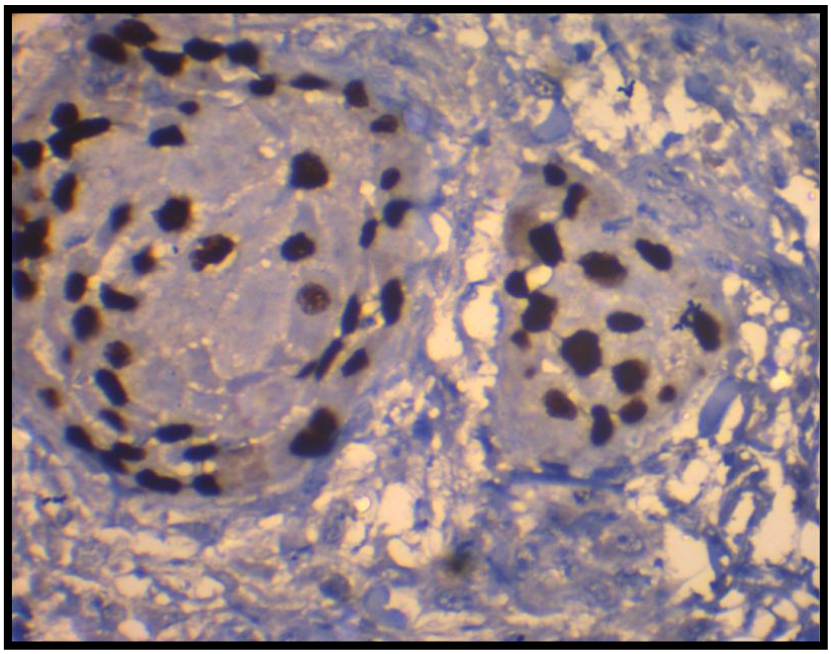
Figure 12(a): Photomicrograph showing H&E stained section showing moderately differentiated carcinoma at low power magnification. **(b&c):** Photomicrograph of IHC stained section showing strong nuclear expression of p63 in moderately differentiated carcinoma at low power magnification (b) & high power magnification(c)



a)



b)



c)

DISCUSSION

Carcinogenesis is a multistep process in which there is a clonal selection and expansion of genetically altered cells[1]. As changes at the molecular level occurs even before any cellular or clinical changes a precise and reliable system to detect these changes will help in early identification of high risk patients and initiate early management thereby reducing the morbidity associated with the lesion[51].

Squamous cell carcinoma is the most common malignancy affecting the oral cavity[1,7]. Most of the cases present with advanced lesions at the time of diagnosis. Histological grade of differentiation & invasion and metastasis & staging determines the prognosis of the patient.

Cell adhesion molecules play an essential role in maintaining the structural integrity of epithelial cells by regulating their growth and differentiation[52]. Reduction in the intercellular adhesion is often associated with loss of epithelial cell differentiation and increases their potential to invade and metastasize. E-Cadherin in association with cytolpasmic regulatory proteins α, β, γ catenin essentially forms the cell junction (Figure 1)[53]. β-catenin also acts as a transcription co-factor in Wnt signaling pathway and forms complex with transcription factor LEF1 in the nucleus[54].

Thus analysing the expression of β-catenin in oral squamous cell carcinoma has a potential to predict the biological behavior of the lesion. Hence we decided to determine objectively the molecular expression of β-catenin in oral squamous cell carcinoma using immunohistochemistry.

In our study previously diagnosed 10 cases of well differentiated carcinoma and 11 cases of moderately differentiated carcinoma were selected. 15 normal tissue samples were selected as controls. Colon cancer was selected as positive control.

Immunohistochemical expression of β-catenin was analyzed based on the intensity of stain uptake and area of epithelial staining. Intensity of staining was scored as nil (0), mild (1) , moderate (2) and intense (3) at high power magnification. Area of epithelial staining was scored as 0 (0%). 1 (<25%), 2 (25-49%), 3 (50-74%) and 4(75-100%) by scanning the entire section. Colon cancer was taken as positive control (Figure 4).

In the normal tissues, β-catenin staining was observed along the cell membrane of basal, parabasal and spinous layer of surface epithelium (Figure 5). Staining was not evident in stratum corneum. A score of 3 was given for the staining intensity for all the normal tissues. Almost all the normal tissue showed more than 75% area of stain uptake by epithelial cells, thus a score of 4 was given excepting 3 cases for which a score of 3 was given.

Membranous expression of β-catenin was found in well differentiated carcinomas (Figure 6). Cytoplasmic localization was evident in few slides. Three cases showed mild staining , three cases showed moderate staining, Intense stain was observed in 4 cases. The area of staining was found to be < 25% in two cases. 25-49% in 2 cases, 50-74% in 4 cases and > 75% in 2 cases.

In moderately differentiated carcinoma (Figure 7) 4 cases showed intense staining, 3 cases showed moderate staining, 2cases showed mild staining and 2 cases showed no staining at all. Area of staining was scored as 4 for 3 cases, 3 for 2 cases, 2 for 3 cases and 0 for 2 cases. In one case no staining was evident in invaded islands (Figure 8).

Comparison of staining intensity showed a significant difference between normal and well differentiated carcinoma and also between normal and moderately differentiated carcinoma. However, no significant difference in staining intensity was found between well differentiated and moderately differentiated carcinoma (Table 2).

Similarly comparison of area of staining showed a significant difference between normal and well differentiated carcinoma and also between normal and moderately differentiated carcinoma. However, no significant difference in area of staining was found between well differentiated and moderately differentiated carcinoma (Table 3).

It is evident that membranous expression of β-catenin is reduced in the case of malignancy compared to the normal tissues. This is similar to the observation made by **Lihong Wand *et al*** [33], **Soichi Iwai *et al*** [34], **Lai-Kui Liu *et al*** [35], **S.Y. Chaw *et al*[5] , Khaled Waleed Zaid *et al*** [9]**.**

APC gene in association with GSK 3β (Glycogen Synthase Kinase) causes degradation of free β-catenin in the cytoplasm by phosphorylation[55]. Mutation in APC gene / GSK 3β / β-catenin phosphorylation site will lead to increase in cytoplasmic accumulation of β catenin[55]. This facilitates β-catenin binding with T cell factor 1-4 and Lymphoid enhancer factor (Lef- 1) and translocate to nucleus. This in turn activates transcription of genes like c-myc, EGFR, urokinase type plasminogen activator receptor tissue proteases etc.. essential for proliferation invasion[56]. This explains why β-catenin was localized along the cell membrane in our normal epithelium.

Wnt secrete lipid modified signaling protein which binds to receptor molecules Frizzled protein and lipo protein receptor related protein 5 &6 (LRP-5/6). This leads to β-catenin accumulation in the cytoplasm. Absence of Wnt signaling keep the levels of β-catenin low by its degradation by APC. Activation of Wnt leads to inhibition of GSK -3 β and thereby APC activity thus there is increased β-catenin in cytoplasm followed by increased transcription in malignancy[57].

This explains the shift in localization of β-catenin from cell membrane to cytoplasm to nucleus in squamous cell carcinoma. The reason for selective nuclear expression of β-catenin in invading islands may be due to the following reason. The increased cytoplasmic β catenin levels leads to activate p53 gene activation[26]. when other mechanism fail to contain the levels of β-catenin in cytoplasm. When the neoplastic cells overcome this counter mechanism, β-catenin translocates to the nucleus followed by transcription of genes related to tissue invasion, proliferation and dedifferentiation of cells[,57,58].

Thus membranous β-catenin expression is found in more differentiated cells while cytoplasmic & nuclear expression seen in less differentiated cells. The basal, para basal and spinous layer expression of β-catenin in epithelium shows their role in maintaining tissue polarity and architecture also.

p53 is a tumor suppressor gene which protects the cells from genotoxic stress by preventing DNA damage. It activates genes p21 which regulates G1 arrest for DNA repair to occur or triggers apoptosis of cells, in case of irreversible DNA damage. Thus it is consider as the guardian of genome. Suppression of P53 function by mutation will lead to carcinogensis[17,59].

Two homologues of p53 namely p63 and p73 have been identified. They share similar transcriptional activation domain, DNA

binding domain and oligomerisation domain similar to p53 (Figure 2), but contain an additional c-terminal extension that is not present in p53[59,60]. They maintain cell homeostasis by interacting with p53 target genes. p63 plays a role in ectodermal differentiation during embryogenesis[61]. It is located on human chromosome 3q27-29[59,62]. Alternative promoters and splicing sites give rise to six different protein isoforms. The transactivating isoforms are TAp63α, TAp63β, TAp63γ. The truncated isoforms lack transactivating domain in N-terminal and includes ΔNp63α, ΔNp63β, ΔNp63γ[59,63].

TAp63 has a role in female germ cell preservation, induces senescence, and protects against cancer metastasis[64]. ΔNp63 has a fundamental role to maintain stem and progenitor in stratified epithelial and glandular tissue. It exerts a negative effect on TAp63 transcriptional activity by repressing TAp63 induced miRNA, thus inhibits senescence and promotes carcinogenesis[65]. Inactivation or loss of TAp63 leads to metastatic and invasive phenotype. p63 isoforms regulate a wide range of target genes with opposite regulatory outcomes[65].

p63 is regulated at mRNA level by RNA binding proteins like PCB1, HuR[59]. Phosphorylation of p63 regulate its activation and degradation. Ubiquitin E3 ligases,kinases are also known to regulate p63 stability[66,67]. The peptidyl –prolyl cis/trans isomerase domain at C

–terminal confers conformational and functional changes of substrate proteins that provides stability to the TAp63α isoforms[68].

TAp63 also regulates metabolism. During starvation of cells it activates glutaminase which converts glutamine to α-ketoglutarate and production of ATP via tri carboxylic acid cycle. The increased glutaminase reduces the ROS levels with in cells. ΔNp63 regulates glutathaione peroxidase 2, an antioxidant Selenium protein which converts hydrogen peroxide to water. Thus isoforms of p63 play an important role in oxidative stress[65].

Loss of TAp63 leads to loss of p53 activity and promotes tumorigenesis. Amplification of p63 genes has been observed in 80% of HNSCC. p63 prevents epithelial mesenchymal transition by inducing mir200. It also suppress metastasis by promoting sharp 1 expression which affects hypoxia induced stability[69,70].

The role of p63, the newer homologue of p53 is not completely known. Hence we decided to study the expression of p63 in different grades of oral squamous cell carcinoma using immunohistochemistry.

In this study previously diagnosed 10 cases of well differentiated carcinoma and 11 cases of moderately differentiated carcinoma was selected. 15 normal tissue samples were selected as controls.

Immunohistochemical expression of p63 was analyzed based on the intensity of stain uptake and area of epithelial staining.

As p63 is expressed in basal cells of normal prostate and absent in prostatic adenocarcinoma normal prostate has been taken as positive control (Figure 9).

Intensity of staining was scored as nil (0), mild (1) , moderate (2) and intense (3) at high power magnification. Area of epithelial staining was scored as 0 (0%). 1 (<25%), 2 (25-49%), 3 (50-74%) and 4(75-100%) by scanning the entire section.

In the normal tissues, nuclear expression of p63 was observed (Figure 10). Staining was not evident in stratum corneum. A score of 3 was given for the staining intensity for nine normal tissues and a score of two for six normal tissues. Six cases showed more than 75% area of stain uptake and was given a score of 4. Six cases obtained a score of three and three case obtained a score of two.

All cases of well differentiated carcinomas showed intense nuclear stains and hence a score of 3 was given (Figure 11). The area of staining was scored as 3 for six cases, two for three cases and four for one case.

Eight cases of moderately differentiated carcinoma (Figure 12) showed intense nuclear stain and were given a score of 3. Two cases showed moderate staining intensity and a score of 2 was given. One case showed mild staining and a score of one was given. The area of staining was found to be more than 75% in 4 cases. Three cases showed 50-74% of staining. Three cases showed, less than 50% of staining and one case showed less than 25% of staining.

Comparison of staining intensity did not show any significant difference between normal and well differentiated carcinoma, between normal and moderately differentiated carcinoma and also between well differentiated and moderately differentiated carcinoma (Table 5).

Similarly comparison of area of staining showed no significant difference between normal and well differentiated carcinoma, between normal and moderately differentiated carcinoma and also between well differentiated and moderately differentiated carcinoma (Table 6).

In all the slides the p63 expression was found in the basal and Spinous layer and it was absent in the corneal layer. Similar observations was made by Nylander et al & Matheny et al. p63 expression was also found in the basal cells of invading islands however the central keratin pearls were unstained (Figure 11). This shows that p63 is associated with the basic proliferative pool and this basic property is retained in the invading islands. Also the observation

that it is evident in spinous layer reveals its role in epithelial stratification. This explains why no difference was observed in the staining intensity and also in the area of staining in normal, well and moderately differentiated carcinomas in our study.

From this we understand that p63 shows a strong nuclear expression both in normal and carcinomas.

Thus we conclude that expression of both β-catenin and p63 can be used as a prognostic marker in oral squamous cell carcinoma.

SUMMARY AND CONCLUSION

The aim of the study was to analyse the immunohistochemical expression of p63 and β-catenin in different grades of oral squamous cell carcinoma. A total of 21 samples of squamous cell carcinoma and 15 samples of normal mucosa were taken from the archival blocks. Immunohistochemical expression of p63 and β-catenin were studied by analysis of intensity and area of staining of these markers.

From the present study following conclusions were drawn:

- Significant difference exists in the intensity and area of staining of β-catenin between normal tissues and carcinoma.
- No significant difference exists in the intensity and area of staining of β-catenin between well and moderately differentiated carcinoma.
- No significant difference exists in the intensity and area of staining of p63 expression between any of the study groups.

We conclude that the reduced expression of β-catenin and shift in the localization from cell membrane to cytoplasm and nucleus has a potential to indicate malignant transformation in the tissues.

The nuclear expression of p63 in normal and malignant tissues suggest that it is closely linked with basal cells and proliferative variant and can be used as potential marker for early diagnosis of carcinoma irrespective of the differentiation status.

Thus we conclude that both expression of β-catenin and p63 can be used as a prognostic marker in oral squamous cell carcinoma.

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ANNEXURE - I

Omsakthi
ADHIPARASAKTHI
DENTAL COLLEGE & HOSPITAL
Recognised by Dental Council of India
Affiliated to The Tamilnadu Dr.M.G.R Medical University
A Unit of Adhiparasakthi Charitable, Medical, Educational & Cultural Trust

This Ethical Committee has undergone the research Protocol submitted by Dr.S.Pradeep Sankar, Post Graduate Student, Department of Oral Pathology & Microbiology, under the title "Immunohistochemical Expression of P63 and β-CATENIN in different grades of Oral Squamous Cell Carcinoma" Ref no : 2016-MDS-BrVI-DEV-14/APDCH under the guidance of Dr.S.Shamala for consideration of approval to proceed with the study.

This Committee has discussed about the Material being involved with the study, the Qualification of the investigator, the present norms and recommendations from the Clinical Research Scientific body and comes to a conclusion that this Research protocol fulfils the Specific requirements and the Committee authorizes the proposal.

Principal

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ANNEXURE - II

OBSERVER SCORES

S.NO	β CATENIN		p63	
	Intensity	Area	Intensity	Area
NORMAL				
1	3	4	3	4
2	3	4	2	3
3	3	4	3	4
4	3	4	3	3
5	3	3	2	2
6	3	4	3	4
7	3	4	2	3
8	3	4	3	4
9	3	4	3	3
10	3	3	2	2
11	3	4	3	4
12	3	4	2	3
13	3	4	3	4
14	3	4	3	3
15	3	3	2	2

WELL DIFFERENTIATED CARCINOMA				
1	1	1	3	3
2	2	2	3	3
3	3	3	3	3
4	1	2	3	3
5	3	4	3	4
6	3	4	3	3
7	3	3	3	2
8	2	3	3	2
9	2	3	3	3
10	1	1	3	2
MODERATELY DIFFERENTIATED CARCINOMA				
1	3	3	3	3
2	3	4	3	3
3	3	4	3	4
4	3	4	3	3
5	2	2	1	1
6	2	2	3	4
7	0	0	2	2
8	0	0	3	2
9	1	2	2	2
10	1	1	3	4
11	2	3	3	4

ANNEXURE - III

URKUND

Urkund Analysis Result

Analysed Document:	MD Pradeep 8.1.19.docx (D46527307)
Submitted:	1/8/2019 7:11:00 AM
Submitted By:	pradeeptindivanam@gmail.com
Significance:	9 %

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